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14. ABSTRACT: The purpose of the research is to identify new ovarian cancer tumor antigens that can be used in the immunotherapeutic treatment of ovarian cancer. The scope of this work involves (1) identifying the peptide antigens recognized by ovarian reactive cytotoxic T lymphocytes (CTL) by using an antigen-unbiased, mass spectrometric approach to antigen identification; and (2) identify peptide antigens within the Her-2/neu, folate binding protein (FBP), and TAG proteins that give rise to ovarian reactive CTL. A total of twelve ovarian cancer lines have been characterized for their expression of class I MHC molecules and tumor antigens. Three TAG-derived epitopes including two HLA-A2-restricted epitopes (SLGWLFLLL and LLLRLECNV) and one HLA-B7-restricted epitope (LPAQEGAPT) were identified. T cell lines are being established from the TAL of ovarian cancer patients. In preliminary experiments, two of these lines appear to recognize the FBP-derived peptide FLLSLALML and the mesothelin-derived peptide FLLFSLGWV. The significance of these results are that they provide new antigens for use in therapeutic vaccine trials for the treatment of ovarian cancer. Furthermore, they provide the reagents necessary to assess the immunologic impact of such trials.

15. SUBJECT TERMS
Ovarian carcinoma, immunotherapy, cytotoxic T lymphocytes, epitope, peptide, major histocompatibility complex (MHC)-encoded molecules, class I MHC molecules, HLA

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INTRODUCTION

The *subject* of this research is vaccine development for the treatment of ovarian cancer. The *purpose* of the research is to identify new ovarian cancer tumor antigens that can be used in the immunotherapeutic treatment of ovarian cancer. Specifically, we will identify the peptide antigens that associate with class I major histocompatibility (MHC) complex coded molecules, and which are capable of stimulating an ovarian cancer cell reactive cytotoxic T lymphocyte (CTL) response. The *scope* of this work involves (1) identifying the peptide antigens recognized by ovarian reactive CTL by using an antigen-unbiased, mass spectrometric approach to antigen identification; and (2) identify peptide antigens within the Her-2/neu, folate binding protein (FBP), and TAG proteins that give rise to ovarian reactive CTL. Although beyond the scope of the present work, it is contemplated that the identified antigens will be tested in future ovarian cancer vaccine trials conducted at the University of Virginia.

BODY

1. Identify the peptide antigens recognized by ovarian reactive CTL by using an antigen-unbiased, mass spectrometric approach to antigen identification (Months 1-36)

1.1. Establish a panel of ovarian cancer cell tumor lines. (Months 1-30)

The ovarian cancer cell line COV413 was acquired from Dr. Angela Zarling at the University of Virginia and added to our collection of ten previously acquired ovarian cell lines. In an ongoing attempt to establish new ovarian cancer cell lines, tumor from five clinical specimens was placed in culture and one of these cultures has yielded a new ovarian cancer line, OAT-11770. Class I MHC typing and analysis by flow cytometry demonstrated that COV413 expresses the HLA-A2 and –B7 class I MHC molecules while OAT-11770 expresses HLA-A2. These lines have also been characterized for the expression of a variety of tumor antigens (Table 1; Appendix 1, Table 1). In addition, we have continued to characterize our previously existing panel of ovarian cancer lines for the expression of four additional tumor antigens including MAGE-A2, MAGE-A4, MAGE-A6, and MAGE-A10 (Table 1).

Folate binding protein (FBP) and Her-2/neu were found to be expressed at high levels in all but the ES-2 ovarian cancer line. The expression of twelve cancer/testis antigens was also studied. One of these, MAGE-A12 was expressed in all twelve ovarian cancer lines and appears to be a promising candidate antigen. MAGE-A1, MAGE-A3, MAGE-A10, NY-ESO-1, TAG-1, TAG-2a, and TAG-2c all have intermediate frequencies of expression and are also good candidates to which to target vaccines. In contrast, TAG-2b is expressed in two lines, MAGE-A2 is expressed in one line, and neither MAGE-A4 or MAGE-A6 is expressed in any of the cell lines.

Although these data are derived from only a total of twelve ovarian cancer lines they illustrate two points. First, some tumor antigens are highly expressed in a significant fraction of ovarian cancer cell lines while others are not. This demonstrates the importance of not only identifying new tumor antigens, but also of determining their frequency of expression so as to identify those have a “practical” significance at the population level. Should these data be representative of ovarian cancer in general, it is obvious, for example, that targeting MAGE-A4 and MAGE-A6 as tumor antigens would be of little therapeutic consequence. Second, each of the ovarian lines expresses a minimum of three different tumor antigens with FBP, Her-2/neu, and MAGE-A12 being the most frequently expressed and at the highest levels. This second point is important as immune escape is less likely to occur when a vaccine targets more than a single antigen on a tumor cell.

1.2. Establish class I MHC-restricted, ovarian cancer cell reactive CTL lines. (Months 1-30)

Tumor associated lymphocytes (TAL) obtained from ascites of four ovarian cancer patients were stimulated with autologous tumor, also derived from the ascites. Following four stimulations with

autologous tumor, the T cell cultures were tested in ^{51}Cr-release assays for their ability to kill autologous tumor, class I MHC-matched tumor, and peptide-pulsed, B-LCL. Characterization of these T cell lines is ongoing as follows.

TAL519 (HLA-A1, -A2, -B7) did not recognize autologous tumor, allogeneic tumor, nor any of the HLA-A1, -A2, or –B7-binding peptides listed in Table 2.

TAL546 (HLA-A3, -B7) shows some evidence of recognition of the autologous tumor as well as the allogeneic ovarian cancer line ES-2 (HLA-A3), although none of the HLA-A3 or –B7 binding peptides from FBP, HER-2/neu or mesothelin (Table 2) were recognized. Experiments are in progress to determine if ES-2 can be used to stimulate the TAL.

TAL572 (HLA-A2) recognizes autologous tumor and possibly the allogeneic ovarian cancer tumor line SKOV3-A2. None of the HLA-A2 binding peptides derived from FBP or mesothelin (Table 2) were recognized. Experiments are in progress to determine if TAL572 recognizes tumor in an HLA-A2-restricted manner.

TAL11770 (HLA-A2) shows some evidence of recognizing the autologous tumor although high background killing on non-HLA matched tumors has made this interpretation difficult. The FBP-derived peptide FLLSLALML, and the mesothelin-derived peptide FLLFSLGWV showed some evidence of recognition and these experiments are being repeated.

An important component of the characterization of the above T cell lines is to determine if the T cell line recognize one or more antigens that are shared between the autologous tumor used to stimulate the response and allogeneic tumor cell lines established from other ovarian cancer patients. Because our goal is to identify antigens that are shared by different tumors it is important to first establish that the T cell line recognizes at least one allogeneic tumor line in addition to the autologous tumor used to stimulate the response.

1.3. Identify the peptide antigens recognized by the CTL established in 1.2. Each identification project will last an average of 4-6 months, and a given antigen identification project is expected to yield from one to several new peptide antigens. (Months 6-36)

As part of each antigen identification project, the T cell line being used is tested for its ability recognize peptide antigens which have previously been synthesized. These peptides might include known peptide epitopes as well as predicted peptide epitopes. If one or more peptides is found to be recognized, then cold target inhibition experiments can be used to determine if the known epitope accounts for all of the recognition. If it does, then identification efforts need not proceed further.

As indicated in 1.2 above, as part of the characterization of the four T cell lines that we are presently working with, we have tested the relevant peptides listed in Table 2. As indicated above there is an initial indication that two peptides might be recognized by the TAL11770 T cell line, but these experiments require confirmation.

Once characterization of the above T cell lines is completed we will be in a position to determine which, if any of them, are appropriate to use for the identification of new antigens. Concurrently, we will continue our efforts to derive additional T cell lines that will be similarly characterized.

2.0 Identify peptide antigens within the Her-2/neu, folate binding protein, and TAG proteins that give rise to ovarian reactive CTL (Months 1-36)

2.1. Predict the Her-2/neu, folate binding protein, and TAG-derived peptides that conform to the HLA-A1, -A2, -A3, -B7, and -B8 binding motifs. Have synthesized the HLA-A1 and –A3 peptides that conform to the rules in specific aim #2. The remaining peptides will be synthesized as needed for step 2.4 below. (Months 1-2)

As indicated in the 1st year of the annual report, all the prediction and synthesis of these peptides has already been accomplished. Because the most cost-effective means of having these peptides was synthesized was to use a multi-peptide synthesis service which synthesizes up to 96 peptides for a single price, we also had synthesized a number of mesothelin-derived peptides as it added no additional costs to the project. Mesothelin has recently emerged as a promsing ovarian cancer antigen (1-3). As was done with the Her-2/neu, FBP, and TAG proteins, the SYFPEITHI and Parker algorithms were used to predict class I MHC binding motifs and the highest ranked peptides were chosen for synthesis. The peptides made are listed in Table 2.

2.2. PCR isolate the cDNA for Her-2/neu and folate binding proteins, and clone the cDNA for Her-2/neu, folate binding protein, and TAG, into the plasmid pcDNA3.1. Plasmids will be transfected as needed. (Months 1-3)

Primers have been designed which amplify the coding region of FBP. The PCR DNA fragment has been isolated and is being cloned into pcDNA3.1 as a *Kpn*I/*Eco*RI insert (Figure 1).

2.3. Stimulate TIL/TAL samples from HLA-A1$^+$ and HLA-A3$^+$ ovarian patients with ovarian peptides predicted to bind to the respective class I MHC molecules. Test the specificity of the ensuing cultures for reactivity with peptide-pulsed target cells and with ovarian cancer cells expressing the appropriate class I MHC molecule and cognate protein. Confirm peptide identity with SRM mass spectrometry. (Months 4-12)

Our initial studies, using PBMC obtained from healthy donors, have concentrated on TAG-derived peptides. These have included three peptides with an HLA-A1 binding motif and two peptides with an HLA-A3 binding motif (Appendix, Table 4). None of these peptides, however, has elicited a peptide-specific CTL response (see Appendix for complete report).

As indicated in section 1.2 above, we have been establishing ovarian cancer reactive CTL lines by stimulating the TAL obtained from ovarian cancer patients with autologous tumor. The CTL that develop in response to autologous tumor are specific for those peptides which are naturally processed and presented by the tumor. By using these CTL lines to screen for reactivity against synthetic peptides, one eliminates the possibility of identifying a peptide that is immunogenic, but not naturally processed and presented. As the tumor of these patients is first screened by PCR to determine if the FBP, Her-2/neu, or TAG antigens are expressed, and because we have class I MHC-typed the PBMC of these individuals, we know when a particular CTL line has the possibility of recognizing peptides that we have predicted to be antigenic.

As an example of the utility of this approach, TAL519 (HLA-A1, -A2, -B7) has been tested against each of the HLA-A1, -A2, and –B7 peptides (25 in total) listed in Table 2. Similarly, TAL546 has been tested against each of the HLA-A3 and –B7 peptides (18 total) listed in Table 2. Although none of these peptides were shown to be recognized, the screening itself was much more rapid than the one we have been using with PBMC obtained from healthy donors.

2.4. Repeat 2.3 for peptides associated with HLA-A2, B7, and B8. The order in which this is done will be dictated by order in which patient material becomes sufficiently available to conduct the experiments. (Months 13-36)

PBMC from healthy donors have also been used as responder cells to identify HLA-A2, -B7, and –B8 restricted peptides derived from TAG. These peptides include four peptides with an HLA-A2 binding motif, five peptides with an HLA-B7 binding motif, and three peptides with an HLA-B8 binding motif (Appendix, Table 4). Peptide-pulsed autologous dendritic cells were used for the primary *in vitro* stimulation, with subsequent stimulations using peptide-pulsed autologous dendritic cells or peptide-pulsed, HLA-matched B-LCL. Three candidate epitopes were identified using this approach and included two HLA-A2-restricted epitopes (SLGWLFLLL

and LLLRLECNV) and one HLA-B7-restricted epitope (LPAQEGAPT). Cytotoxic T lymphocytes specific for each of these peptides were capable of recognizing tumor cells expressing both the corresponding class I major histocompatibility complex encoded molecule and the TAG genes. The tumors recognized were melanomas (Appendix 1; Figure 5); ovarian cancer tumor lines were not recognized. This latter result suggest that either expression as detected by PCR does not correlate with protein expression or that the lines might have antigen processing defects. We are in the process of producing antisera to the TAG protein and should be able to address this question in the near future.

An important outgrowth of these studies was a technical development that allows for the simultaneous screening of up to four peptides. While the standard protocol for these experiments involves stimulating donor PBMC with a single peptide, the modified protocol utilizes stimulations with up to four different peptides (Appendix, Table 4). Following multiple rounds of stimulation, the T cell cultures are tested against a pool of all the peptides, with those cultures exhibiting reactivity subsequently tested against the individual peptides. The utility of this approach is demonstrated with the HLA-A2 restricted peptide, SLGWLFLLL, which was identified as antigenic using both the original and modified approach (Appendix, Table 4).

TAL572 (HLA-A2) and TAL11770 (HLA-A2) were stimulated with autologous tumor as described in 2.3 above. The ensuing CTL lines were tested against the FBP and mesothelin peptides listed in Table 2. Preliminary experiments suggest that the FBP-derived peptide FLLSLALML and the mesothelin-derived peptide FLLFSLGWV might be recognized by these CTL. Experiments are in progress to confirm this.

2.5 Determine the ability of CTL generated in 1.2 above to recognize target cells transfected or infected with the gene of interest and identify the peptide antigen. Confirm with SRM mass spectrometry. (Months 6-36)

This work will be initiated during the upcoming year.

Table 1. Tumor Antigen Expression in Ovarian Cancer Lines

Line	MHC-I	TuAg	FBP	Her-2/neu	MAGE-A1	MAGE-A2	MAGE-A3	MAGE-A4	MAGE-A6	MAGE-A10	MAGE-A12	NY-ESO-1	TAG-1	TAG-2a	TAG-2b	TAG-2c
CAOV-3	A69	PCR	++++	++++	-	-	-	-	-	-*	+++	++	++	+	-	-
CA0V-4	A2	PCR	++++	++++	-	-	-	-	-	-*	+++	-	+	-	-	-
COV413	A2; B7	PCR	+++	++++	-	-	-	-	-	-*	+++	++	-	+	-	-
ES-2	A3, 68	PCR	+	+	+++	-	++++	-	-	+++*	++++	++	+	-	-	-
OV-90	A2	PCR	++++	++++	+++	-	++++	-	-	+++*	++++	+++	++	++	-	-
OVCAR3	A2; B7	PCR	++++	++++	-	-	-	-	-	++++*	+++	++	++++	++++	+++	++++
SK-OV-3	A3, 68	PCR	++++	++++	+++	-	++	-	-	-*	+++	++	+	-	-	-
SW626	A3; B7	PCR	++++	++++	-	-	+	-	-	-*	+++	++	++	++	-	+
TOV-21G	-	PCR	++++	++++	-	+	-	-	-	-*	+++	-	+	-	-	-
TOV-112D	A3	PCR	+++	++++	-	-	+	-	-	-*	+++	++	-	-	-	-
TTB-6	A2, 68?	PCR	++++	++++	-	-	-	-	-	-*	+++	++	++++	++++	+++	++++
OAT-11770	A2	PCR	++++	++++	-*	-	-*	-	-	-*	+++*	-*	-*	-*	-*	-*

PCR Key:

++++ easily visible at 30 cycles
+++ weakly visible at 30 cycles, easily visible at 40 cycles
++ not visible at 30 cycles, easily visible at 40 cycles
+ not visible at 30 cycles, weakly visible at 40 cycles
- not visible at 30 cycles, not visible at 40 cycles
-

Data that is “greyed" is from a single experiment and needs to be repeated.

Table 2. Peptides Synthesized for Testing[a]

Source Protein	Tested Peptides Segregated by Class I MHC Binding Molecule			
	HLA-A1	HLA-A2	HLA-A3	HLA-B7
FBP	PNEEVARFY	LLLVWVAVV FLLSLALML LLSLALMLL SLALMLLWL	LLNVSMNAK RVLNVPLSK YLYRFNWNH AVVGEAQTR	GPWAAWPFL AQRMTTQLL WPFLLSLAL
HER-2/neu	HLDMLRHLY LLDIDETEY VSEFSRMAR		ILIKRRQQK ILKETELRK	DVRLVHRDL LPASPETHL
Mesothelin	EIDESLIFY TLDTLTAFY	FLLFSLGWV SLLFLLFSL VLPLTVAEV	ELAVALAQK ALQGGGPPY	APTEDLKAL RVRELAVAL GPGPVLTVL GPVLTVLAL
TAG-1		LLLRLECNV SLGWLFLLL		LPAQEGAPT

[a]Each protein sequence was analyzed with the SYFPEITHI and Parker algorithms. The highest ranked peptides were selected and synthesized.

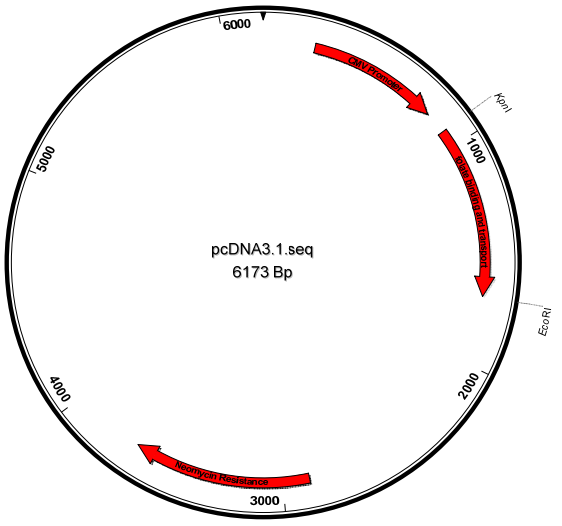

Figure 1. FBP gene construct in pcDNA3.1.

KEY RESEARCH ACCOMPLISHMENTS

- OAT11770 was established as a new ovarian cancer cell line. A total of twelve ovarian cancer lines have been characterized for their expression of class I MHC molecules and tumor antigens. This characterization greatly increases the utility of these cell lines for immunological studies, both as stimulator cells to generate CTL, and as target cells for the specificity analysis of CTL. The lack of such characterized cells in the past has been an impediment to both the execution and interpretation of experiments designed to investigate the T cell mediated immune response to ovarian cancer.
- Further refined the methodology for assessing the immunogenicity of peptides predicted to be presented in association with define class I MHC molecules such that up to four peptides can be simultaneously tested.
- Three TAG-derived epitopes including two HLA-A2-restricted epitopes (SLGWLFLLL and LLLRLECNV) and one HLA-B7-restricted epitope (LPAQEGAPT) were identified using CTL derived from the PBMC of healthy donors. CTL specific for each of these peptides are capable of recognizing tumor cells expressing both the corresponding class I MHC complex-encoded molecules and the TAG genes. As TAG-1 is expressed in 75% of twelve ovarian cancer cell lines and 35% of 23 fresh ovarian cancer patient samples, these peptides are reasonable candidates for inclusion in future ovarian cancer vaccines designed to stimulate a therapeutic CTL response. A caveat to this finding is that the peptide-specific CTL recognized melanoma, but not ovarian tumor lines. The lack of recognition of the ovarian tumor lines could be due to a defect in antigen processing/presentation or a lack of expression of the TAG protein at the protein, but not mRNA level. Additional work is required to distinguish between these possibilities. This work is reported in the submitted manuscript referenced below.
- T cell lines are being established from the TAL of ovarian cancer patients. In preliminary experiments, two of these lines appear to recognize the FBP-derived peptide FLLSLALML and the mesothelin-derived peptide FLLFSLGWV.

REPORTABLE OUTCOMES

Patent Application

Hogan, K.T. TAG-derived epitopes and uses thereof. US Provisional Patent Application Serial No. 60/856,510 filed on November 3, 2006.

Submitted Manuscript

Adair, S.J., Carr, T.M., Fink, M. J., Slingluff, C. L., and Hogan, K. T. (2006) The TAG family of cancer/testis antigens is widely expressed in a variety of malignancies and gives rise to HLA-A2 and HLA-B7-restricted epitopes. Cancer Immunol. Immunotherapy. Submitted.

Ovarian Cancer Cell Lines Established

OAT11770 was established.

CONCLUSION

The importance of these findings are three-fold. First, a panel of twelve ovarian cancer cell lines has been established and characterized for the expression of class I MHC molecules and a variety of tumor antigens. Having such a panel is instrumental in conducting these studies and will also be a major resource in trying to interpret the results of ovarian cancer vaccine clinical

trials. Second, three novel peptide antigens have been identified and two additional peptide antigens have been tentatively identified. These peptides are of potential utility in future vaccine trials for the treatment of ovarian cancer. Third, we have developed and refined the necessary protocols to produce CTL for the identification of additional peptide antigens.

The identification of additional antigens that can be used in a therapeutic vaccine for the treatment of ovarian cancer is medically important because there are relatively few antigens that are currently available for such a vaccine. One consequence of this limitation is that if a patient's tumor does not express at least one of the antigens in the vaccine, the vaccine cannot induce a therapeutic effect even if it can stimulate a CTL response. Practically speaking, this means that many patients will be excluded from vaccine trials that measure clinical endpoints as there is no likelihood of a clinical benefit. The three peptide antigens identified here, and those that will be identified in the future will expand the percentage of women likely to benefit from therapeutic vaccination. Additional peptide antigens in a vaccine also provide two additional theoretical advantages. First, the more antigens towards which the response is directed, the less likely it will be that antigen loss on the tumor will lead to escape from the elicited CTL response. Second, responses against multiple antigens will increase the magnitude of the response and increase the likelihood that the ensuing CTL response will be clinically effective.

The development of a well-characterized panel of ovarian cancer cell lines and the identification of peptide antigens specific for ovarian cancer is scientifically important as it provides the necessary resources to define *in vitro* end points for future ovarian cancer vaccine trials.

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APPENDIX

Adair, S.J., Carr, T.M., Fink, M. J., Slingluff, C. L., and Hogan, K. T. (2006) The TAG family of cancer/testis antigens is widely expressed in a variety of malignancies and gives rise to HLA-A2 and HLA-B7-restricted epitopes. Cancer Immunol. Immunotherapy. Submitted.

The TAG family of cancer/testis antigens is widely expressed in a variety of malignancies and gives rise to HLA-A2 and HLA-B7-restricted epitopes

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Keywords: Cancer/testis antigen, CTL, Epitope, Immunotherapy

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Abbreviations: B-LCL: B-lymphoblastoid cell line · C:H: Cold-to-Hot · CTL, Cytotoxic T Lymphocyte · E:T: Effector-to-Target · mDC: Mature Dendritic Cells · FBS: Fetal Bovine Serum · mAb: Monoclonal Antibody · MHC: Major Histocompatibility Complex · PBL: Peripheral Blood Lymphocytes · PBMC: Peripheral Blood Mononuclear cells · PCR: Polymerase Chain Reaction

Abstract The TAG-1, TAG-2a, TAG-2b, and TAG-2c cancer/testis genes, known to be expressed in an unusually high percentage of melanoma cell lines, are shown here to be expressed in a variety of tumor lines of diverse histological type including cancers of the brain, breast, colon, lung, ovary, pharynx, and tongue. The genes are also expressed in fresh, uncultured melanoma and ovarian cancer cells. Epitope prediction algorithms were used to identify potential HLA-A1, -A2, -A3, -B7, and -B8 epitopes, and these potential epitopes were tested for their ability to stimulate a peptide-specific cytotoxic T lymphocyte response using lymphocytes from healthy donors. Three candidate epitopes were identified using this approach and included two HLA-A2-restricted epitopes (SLGWLFLLL and LLLRLECNV) and one HLA-B7-restricted epitope (LPAQEGAPT). Cytotoxic T lymphocytes specific for each of these peptides were capable of recognizing tumor cells expressing both the corresponding class I major histocompatibility complex encoded molecule and the TAG genes. These results indicate that TAG-derived peptides would be good components of a therapeutic vaccine designed to target melanoma and a variety of epithelial cell-derived malignancies.

Introduction

Cytotoxic T lymphocyte (CTL) mediated cytotoxicity and cytokine secretion have emerged as major mechanisms by which tumor growth is controlled by the mammalian immune system (1). Vaccination of mice with immunogenic peptides has been shown to control tumor growth in both therapeutic and prophylactic models of cancer (2, 3). Vaccine trials have begun in humans, with most efforts using antigenic peptides known to bind to class I major histocompatibility complex (MHC) molecules, although class II MHC molecule binding peptides are also being tested (4-7). Although a substantial number of peptides have been discovered that can be used for the treatment of melanoma, there are relatively fewer peptide antigens that can be used for the treatment of other malignancies (8-10). Identification of additional peptide antigens would expand the number of malignancies that are amenable to vaccine-mediated therapy, and the

use of a large number of peptides in a vaccine would minimize the impact of antigen loss variants that arise in the presence of immunoselection (11-14).

The categories of proteins giving rise to the tumor antigens recognized by CTL include cancer/testis antigens, differentiation antigens, mutated gene products, widely expressed proteins, and viral proteins (15-17). Cancer/testis antigens are particularly attractive candidates for use in tumor vaccines, as these antigens are only expressed in the testis and occasionally the placenta, which are both immunologically privileged sites (18). A consequence of this pattern of expression is that the peripheral CTL are not rendered tolerant to cancer/testis antigens and can thus recognize the antigens when they are expressed on tumor cells. The cancer/testis antigen family now contains a wide variety of proteins, prototypic members of which are exemplified by the MAGE (19) and NY-ESO-1 (20) antigens. More recently, we have identified multiple isoforms (TAG-1, TAG-2a, TAG-2b, TAG-2c, and TAG-3) of a gene coding for a new cancer/testis antigen (21).

The TAG-1, -2a, -2b, and -2c genes were previously shown to be expressed in almost 90% of thirty-two melanoma lines tested (21). Unlike most cancer/testis antigens which are rarely expressed in leukemia or myeloma cells, the TAG genes were also found to be expressed in K562, a myelogenous leukemia, and they were found to be homologous with chronic myelogenous leukemia-derived clones in the human EST database (21). Importantly, TAG was shown to be naturally immunogenic as the TAG-derived peptide RLSNRLLLR was shown to be recognized by HLA-A3-restricted CTL obtained from a melanoma patient (21, 22). We have expanded on this work in the present study by demonstrating that the TAG gene is expressed in a variety of epithelial cell-derived tumors and by identifying additional TAG-derived peptides that elicit tumor-reactive CTL responses.

Materials and Methods

Growth medium

RPMI-1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (complete RPMI) served as the base medium. Base medium was supplemented with 10% fetal bovine serum (RPMI-10FBS) or 10% human serum (RPMI-10HS). For the growth of some tumor lines RPMI-10FBS was additionally supplemented with 1 mM sodium pyruvate, 5 ng/ml epidermal growth factor, 10 µg/ml transferrin, and 7.3 µg/ml insulin (RPMI-10FBS-PETI).

Cell lines

The brain cancer lines SW-1088, T98G, U-87MG, and U-373MG were obtained from the ATCC (Manassas, VA). The breast cancer lines MCF7, MDA-MB-453, MDA-MB-468, and SK-BR-3 were obtained from the ATCC; the breast cancer lines VAB5-A, BRC-173, and BRC-751 were established at the University of Virginia. The colon cancer lines HT-29, LS174T, and SW480 were obtained from the ATCC; the colon cancer line VCR-8 was established at the University of Virginia. The lung cancer lines Calu-1, SK-LU-1, and SK-MES-1 were obtained from the ATCC; the lung cancer lines TTB-250, VBT-2, VLU-6, VLU-13, VLU-18, and VLU-19 were established at the University of Virginia. The ovarian cancer lines CAOV-3, CAOV-4, ES-2, OV-90, OVCAR3, SK-OV-3, SW626, TOV-21G, and TOV-112D were obtained from the ATCC. The ovarian cancer cell line TTB-6 was established at the University of Virginia, and the ovarian cancer cell line COV413 was obtained from Dr. Angela Zarling (University of Virginia). The squamous cell carcinomas of pharyngeal (FaDu), tongue (SCC4) and cervical (SiHa) origins were obtained from the ATCC. The cancer lines were maintained in RPMI-10FBS or RPMI-10FBS-PETI.

The B-lymphoblastoid cell lines (B-LCL) JY, MST, and T2 were maintained in RPMI-10FBS. C1R-A2, C1R-A3, and C1R- B7 were maintained in RPMI-10FBS supplemented with 300 µg/ml G418.

Patient material

Cryopreserved tumor digest was obtained from the Tissue Procurement Facility at the University of Virginia. The tissue was obtained in an anonymized fashion and in accordance with established Institutional Review Board protocols.

Polymerase chain reaction (PCR)

Total RNA was prepared from 2-10 x 10^6 cells using the RNeasy® Mini kit (Qiagen, Valencia, CA) as per the kit instructions. RNA was quantified by absorbance at 260 nm. Total RNA was converted to cDNA by using the SuperScript™ First-Strand Synthesis System (Invitrogen, Carlsbad, CA). Previously designed primers were used to amplify GAPDH (1361, 1362; 598 bp), TAG-1 (A52, C723; 672 bp), TAG-2a (A73, E600; 528 bp), TAG-2b (A73.92, F473; 401 bp), and TAG-2c (A73, G608; 536 bp) (21). PCR was performed on 250 ng of cDNA using Platinum *Taq* High Fidelity (Invitrogen). The PCR mixes were heated to 94°C for 2 min, 30 and 40 cycles of amplification were performed, and a final extension completed at 68°C for 5 min. When amplifying the TAG genes, the 30 and 40 cycles consisted of 94°C for 30 s, 62°C for 30 s, and 68°C for 60 s. When the GAPDH gene was amplified, the 30 cycles consisted of 94°C for 30 s, 60°C for 30 s, and 68°C for 60 s. The PCR products were visualized on ethidium bromide-stained agarose gels.

Epitope prediction

The TAG-1 and TAG-2 genes can each be potentially translated into three different isoforms (TAG-1α, TAG-1β, TAG-1γ, TAG-2 α, TAG-2 β, and TAG-2γ), with the putative α, β, and γ forms differing in the length at the N-terminal end of the protein. For the purposes of epitope prediction the largest isoforms of each, TAG-1α and TAG-2α, were used and are referred to herein as TAG-1 and TAG-2.

The SYFPEITHI [www.syfpeithi.de] (23) and Parker [bimas.cit.nih.gov/molbio/hla_bind] (24) epitope prediction algorithms were used to identify peptides that have a high predicted binding affinity for HLA-A1, -A2, -A3, -B7, and -B8. The peptides were synthesized (New England Peptide, Inc., Gardner, MA) and resuspended at 2-10 µg/ml in 100% dimethyl sulfoxide.

Peripheral blood mononuclear cells (PBMC)

The buffy coat fraction from a unit of blood was obtained as a byproduct of voluntary blood donations by healthy individuals (Virginia Blood Services, Richmond, VA). Peripheral blood mononuclear cells (PBMC) were obtained following centrifugation on Ficoll-Paque™ PLUS (Amersham Biosciences, Uppsala, Sweden) and washed twice. Monocytes were enriched from the PBMC by adherence to tissue culture flasks for 2 h and were subsequently used for the generation of dendritic cells. The plastic non-adherent cells were used as peripheral blood lymphocytes (PBL) and were cryopreserved until use.

Class I MHC gene typing

DNA was obtained from 5 x 10^6 PBMC using the DNeasy® Tissue Kit (Qiagen) according to the manufacturer's instructions. Class I MHC typing was done using the Micro SSP™ Generic HLA Class I typing tray SSP1L (One Lambda, Inc., Canoga Park, CA).

Dendritic cell preparation

Dendritic cells were prepared using a modification of a previously published protocol (25). In brief, adherent monocytes were incubated for 6-8 d in the presence of 800 U/ml GM-CSF and 500 U/ml IL-4 to produce immature dendritic cells. The non-adherent, immature dendritic cells were then incubated in the presence of 800 U/ml GM-CSF, 500 U/ml IL-4, 10 ng/ml IL-1β, 1,000

U/ml IL-6, 10 ng/ml TNFα, and 1 μg/ml PGE_2. Mature dendritic cells (mDC) were obtained as non-adherent cells following 3-4 d of incubation. mDC were incubated with peptide (40 μg/ml) and β_2-microglobulin (3 μg/ml) for 2 h at room temperature. The peptide-pulsed mDC were irradiated (3,500 Rad) and washed once to remove free peptide. The cells were then used immediately for CTL stimulation or were cryopreserved for future stimulations.

Stimulation of peptide-specific CTL

CTL were stimulated using a modification of the protocol of Lu and Celis (26, 27). Equal volumes of PBL (2 x 10^6 cells/ml) were mixed with autologous, peptide-pulsed mDC (1 x 10^5 cells/ml) to give a responder to stimulator ratio of 20:1 in RPMI-10HS supplemented with 10 ng/ml IL-7. Wells (generally 48) on a 48-well plate were seeded with 0.5 ml of the mixed responder/stimulator cells. One day following the initial priming, IL-10 at a final concentration of 10 ng/ml was added to each well. The cultures were restimulated every 7 days. For secondary stimulations, peptide-pulsed stimulator cells were added to each well in 0.5 ml RPMI-10HS. IL-10 (10 ng/ml) was added one day later, and IL-2 at a final concentration of 10 Cetus U/ml was added two days later. Two to three days later IL-2 (10 Cetus U/ml) was added again to each culture. Tertiary and later stimulations were done in a similar fashion except that IL-2 was the only added cytokine. For all stimulations, 2.5 x 10^4 mDC were used as the stimulator cells in the restimulations until they were depleted, after which 1 x 10^6 autologous PBL or 2.5 x 10^5 to 5.0 x 10^5 B-LCL matched for the class I MHC molecule of interest were used. T cell cultures showing activity against peptide-pulsed targets in a screening assay were expanded with anti-CD3 (28).

Cytotoxicity assays

A standard 4 h ^{51}Cr-release assay was used and modified as indicated below for the indicated analyses (29). Target cells were labeled with 100 µCi $Na_2{}^{51}CrO_4$ for 2 h and then washed. The

^{51}Cr-labeled cells were then incubated with peptide (10 µg/ml) for 1 h at room temperature, washed, and added to wells containing CTL on a 96-well plate at 2,000 cells/well. Maximal ^{51}Cr-release was determined by incubating labeled target cells in the presence of 3% NP-40, and spontaneous ^{51}Cr-release was obtained by incubating with assay medium alone. Counts per minute (CPM) present in the collected supernatants were measured using a Wallac WIZARD automatic gamma counter (Perkin Elmer, Downers Grove, IL). The percent specific release was calculated as: % specific ^{51}Cr-release = 100*(ER-SR)/(MR-SR) where ER = CPM experimental release, SR = CPM spontaneous release, and MR = CPM maximal release.

Cytotoxicity screening assay. T cell cultures were tested for cytotoxic activity against peptide-pulsed cells five to six days following the fourth stimulation. Cells from four to six randomly selected wells from each 48-well plate to be tested were counted and averaged to obtain a mean effector cell concentration. Average effector-to-target (E:T) ratios of 40:1 and 10:1 were tested in duplicate for each culture. In some experiments unlabeled K562 cells were added at an unlabeled-to-labeled target ratio of 20:1 to decrease non-specific killing attributable to NK cells.

CTL specificity analysis. ^{51}Cr-labeled tumor cells and peptide-pulsed B-LCL were used as targets. CTL cultures were counted and resuspended at a concentration giving an initial E:T ratio which generally ranged from 20:1 to 80:1, with subsequent 1:2 dilutions used to give four E:T ratios for each CTL being analyzed.

Peptide titration. Peptides, starting at 10 µg/ml, were diluted through a ten-fold dilution series, after which ^{51}Cr-labeled targets were added for 1 h at room temperature. The target cells were then resuspended at 20,000 cells/ml and added to the appropriate wells of the 96-well plates in 100 µL aliquots. Effector cells were then added at 100 µL/well to give the desired E:T ratio.

Cold target inhibition. Cold targets, starting at a 40:1 cold-to-hot (C:H) ratio were diluted two-fold in a final volume of 50 µl per well. Cold target cells, when appropriate, were first incubated with peptide (0.1-10 µg/ml depending on avidity) for 1 h at room temperature. Before plating, peptide-pulsed cold target cells were washed with 10x volume of RPMI 1640 containing 1% FBS. Effector cells, in a volume of 50 µl/well, were then added. The 96 well plates were then centrifuged at 800 rpm for 2 m at room temperature and incubated at 37°C for 1 h. After the incubation, ^{51}Cr-labeled targets were added in 100 µl aliquots. Each cell line used in the assay was also evaluated in triplicate, without cold targets, at the appropriate E:T ratios.

Human subjects

All research involving human subjects and human tissues was approved by the University of Virginia Institutional Review Board in accordance with an assurance filed with and approved by the Department of Health and Human Services.

Results

Expression of TAG genes in established tumor lines

The TAG genes were previously shown to be expressed in a large percentage of melanoma cell lines (21). To determine if the TAG genes are expressed in other cancers, a broad survey was taken using established tumor lines of non-melanocytic origin. Brain tumors, which share an ectodermal origin with melanocytes, expressed the TAG-1, TAG-2a, and TAG-2c genes, but not the TAG-2b gene (Table 1). Tumor lines of epithelial cell origin including breast, colon, lung, ovarian, pharyngeal, tongue and cervical origin were also tested for expression of the TAG genes (Table 1). With the exception of the pharyngeal, tongue, and cervical tumor lines for which only one cell line each was tested, each of the TAG genes was expressed in at least one line of each cancer type. As with melanoma (21), TAG-1 was the most frequently expressed TAG gene while TAG-2b was the least frequently expressed TAG gene. By performing the PCR

amplification at both 30 and 40 cycles, it was also possible to categorize the expression levels in individual cell lines. The data demonstrate that the TAG genes are strongly expressed in some tumor lines while weakly expressed in others. (Table 1). Because a strong positive amplification signal of the GAPDH gene was obtained for each of the cDNA samples (data not shown), the lack of TAG gene amplification for a given sample cannot be attributed to the quality of the cDNA preparation.

Expression of TAG genes in uncultured melanoma and ovarian carcinoma cells

It was previously shown that the TAG genes were expressed in the uncultured melanoma cells from which the VMM12 melanoma line was established and from which the TAG genes were originally cloned and identified, thus demonstrating that the expression of the genes is not an artifact of *in vitro* cell culture (21). That work has been extended here by showing that the TAG genes are expressed in a relatively high frequency of uncultured melanomas and ovarian carcinomas (Table 2). As with the tumor lines, TAG-1 is the most frequently expressed and TAG-2b is the least frequently expressed TAG gene.

Prediction of class I MHC binding peptides from TAG-1 and TAG-2

The SYFPEITHI (23) and Parker (24) epitope prediction algorithms were used to predict HLA-A1, -A2, -A3, -B7, and -B8 binding peptides from the TAG-1 and TAG-2 proteins (Table 3). The top five peptides predicted by each algorithm were reviewed and those peptides not containing canonical anchor residues were eliminated. The remaining two to five top ranked peptides were then selected for further synthesis and testing. The peptide RLSNRLLLR was predicted to bind HLA-A3 (ranking in the top 1-4 scores for TAG-1 and TAG-2), but was not chosen for study as we previously demonstrated that it is recognized by a tumor-reactive CTL line that naturally developed in a melanoma patient (21).

Generation of CTL specific for TAG-derived peptides

Each peptide listed in Table 4 was synthesized and tested for its ability to prime a peptide-specific CTL response using PBL obtained from three to nine healthy donors. PBL were stimulated with peptide-pulsed, autologous mDC, generally in 48 individual microcultures per donor. Following the fourth restimulation, the individual cultures were tested for reactivity with peptide-pulsed target cells in a ^{51}Cr-release assay. Cultures, with killing that was more than 20% above that found on the target cells not pulsed with peptide, were selected for additional characterization. Initially, individual microcultures were stimulated with only a single peptide, and these cultures identified two peptides for further study including the HLA-A2-restricted peptide SLGWLFLLL and the HLA-B8-restricted peptide LSRLSNRLL (Table 4). Selected cultures reactive with SLGWLFLLL and LSRLSNRLL were expanded with anti-CD3 antibody for further analysis. The reactivities of six SLGWLFLLL cultures are shown in Fig. 1. The culture reactive with the LSRLSNRLL peptide lost its peptide-specificity following expansion, and although it recognized tumor, it was not studied further.

Screening individual test peptides for their ability to stimulate a CTL response is an inherently time and resource intensive endeavor. To determine the feasibility of screening multiple peptides simultaneously, PBL of one of the donors reactive against the SLGWLFLLL peptide in association with HLA-A2 was also stimulated with a mix of four peptides including SLGWLFLLL. Because of limitations on the number of responder cells in each microculture, the initial screening was performed against targets pulsed with all four peptides, and this yielded positive cultures (Table 4). Cultures positive for cytotoxic reactivity were then expanded with anti-CD3 and tested against targets individually pulsed with each of the peptides used in the stimulation. Not only was a response against SLGWLFLLL in the mix detected, but a response was also measured against LLLRLECNV in association with HLA-A2, thus validating that

multiple peptides can be tested simultaneously for their ability to induce a CTL response (Table 4; Fig. 2A, C). A similar peptide mix lacking the SLGWLFLLL peptide was also used to identify another LLLRLECNV reactive culture in an additional donor (Table 4). Likewise, a mix of HLA-B7 peptides led to the identification of LPAQEGAPT as a candidate epitope (Table 4; Fig. 2B, D).

MHC restriction of the SLGWLFLLL, LLLRLECNV, and LPAQEGAPT peptides

To determine whether the SLGWLFLLL and LLLRLECNV peptides are presented by HLA-A2 and whether the LPAQEGAPT peptide is presented by HLA-B7, the respective CTL cultures were tested against a panel of target cells that were either matched or unmatched for the class I MHC molecule of interest. The target cells were incubated in the presence of the test peptide and then tested for their susceptibility to lysis by the peptide-specific CTL. CTL line 22E5, specific for the SLGWLFLLL peptide, recognized the HLA-A2 positive cell lines T2 and C1R-A2 when pulsed with peptide, but did not recognize the HLA-A2 negative MST and C1R-B7 lines when pulsed with peptide (Fig. 3A). The same results were obtained with the CTL line 82C8, specific for the LLLRLECNV peptide (Fig. 3B). These results demonstrate that both the SLGWLFLLL and LLLRLECNV peptides are presented by HLA-A2.

CTL line 65F2, specific for the LPAQEGAPT peptide, recognized the HLA-B7 positive cell lines C1R-B7 and MST when pulsed with peptide, but did not recognize the HLA-B7 negative T2 and C1R-A2 lines when pulsed with peptide (Fig. 3C). These results demonstrate that that the LPAQEGAPT peptide is presented by HLA-B7.

Peptide Dose-Response of Peptide-Specific CTL

The relative affinity of the peptide-specific CTL lines was determined by testing the ability of the CTL to recognize target cells incubated with 10-fold dilutions of peptide, beginning at 10 µg/ml

(~10 μM). CTL lines recognizing the SLGWLFLLL peptide showed a broad range of peptide concentrations over which half-maximal killing was achieved, with most having half-maximal activity between 0.1 and 10 nM (Fig. 4A). CTL lines recognizing the LLLRLECNV peptide had half maximal activity between 1 and 20 nM (Fig. 4B), while CTL lines recognizing the LPAQEGAPT peptide required between 20 and 1000 nM peptide concentrations for half-maximal sensitization (Fig. 4C).

Recognition of Tumors by Peptide-Specific CTL

To determine if the peptide-specific CTL also recognize tumor cells, the CTL were tested for their ability to recognize tumors expressing both the appropriate class I MHC molecule and the TAG gene. CTL lines 22E5 and 69C4 (SLGWLFLLL specific) recognized some, but not all, tumors expressing both HLA-A2 and the TAG genes, however, tumors expressing either HLA-A2 or the tumor antigen alone were not recognized (Fig. 5A,B, and data not shown). Likewise, CTL line 65F2 (LPAQEGAPT specific) recognized VMM12 expressing both HLA-B7 and the TAG genes, but not tumors expressing either HLA-B7 or the tumor antigen alone (Fig. 5C). Finally, CTL line 82C8 (LLLRLECNV specific) recognized some, but not all tumor lines expressing HLA-A2 and the TAG genes, however, tumors expressing either the HLA-A2 or the tumor antigen alone were not recognized (Fig. 5D).

The tumor reactivity of the CTL lines was further confirmed in cold target inhibition experiments. When unlabeled DM6 and SLGWLFLLL peptide-pulsed T2 cells were used as cold targets they inhibited the recognition of SLGWLFLLL peptide-pulsed T2 cells by the 22E5 CTL line (Fig. 6A) Similarly, unlabeled DM6 and LLLRLECNV peptide-pulsed T2 inhibited the recognition of DM6 tumor cells by CTL line 82C8. (Fig. 6B). Reciprocal inhibition was obtained with CTL line 82C8 when LLLRLECNV peptide-pulsed T2 cells were used as the hot targets and DM6 tumor cells were used as the cold target (data not shown). These results confirm that the

SLGWLFLLL and LLLRLECNV peptides are presented by HLA-A2 on DM6 tumor cells. Conversely, when unlabeled SLGWLFLLL peptide-pulsed T2 cells were used as inhibitors of ^{51}Cr-labeled DM6 tumor cells, a reproducible enhancement in killing of DM6 by CTL line 22E5 was observed (data not shown), presumably due to a carryover of peptide from the peptide-pulsed cells to the tumor cells.

Cold target inhibition experiments performed with LPAQEGAPT-specific CTL line 65F2 demonstrated that both VMM12 and LPAQEGAPT-pulsed C1R-B7 inhibited the recognition of LPAQEGAPT-pulsed targets, thus demonstrating that the peptide epitope is shared on VMM12 tumor cells (Fig. 6C). The reciprocal experiment in which VMM12 was used as the hot target resulted in no change or an enhancement in the recognition of the tumor cells (data not shown). As with CTL line 22E5, this was presumably due to peptide carryover to the tumor.

Discussion

Cancer/testis antigen expression has most often been studied at the mRNA level by PCR analysis (30, 31). In melanoma, mRNA expression for different cancer/testis antigens ranges from about 4 to 90% (21, 30). TAG (~90%) and MAGE-3 (~50-90%) are the most frequently expressed cancer/testis antigens, while SAGE is infrequently expressed (4%). Many cancer/testis antigens including BAGE, CT7, GAGE, MAGE-1, NY-ESO-1, and SSX-2 are expressed in about 25-75% of the melanoma samples tested. In a study of eight cancer/testis antigens in 47 different melanoma tumors, 91% expressed at least one of the antigens, and 13% expressed all of the antigens (30). The present work extends our previous findings with TAG in melanoma by demonstrating that the family of genes is expressed in 23% to 59% of 22 fresh melanomas tested (Table 1). Although this level of expression is somewhat less than that found in cultured melanoma cell lines, it is still a relatively high frequency of expression in comparison to other cancer/testis antigens.

As cancer/testis antigens are expressed in a wide variety of cancers of diverse histological types (18), it was also of interest to determine if tumors of epithelial origin expressed the TAG genes. Established tumor lines including breast, colon, lung, pharyngeal, and tongue all expressed TAG, with TAG-1 being the gene most often expressed and at the highest levels (Table 1). A similar finding was also observed with brain tumors which, like melanoma cells, are of an ectodermal lineage (Table 1). As is the case with uncultured melanoma cells, the TAG genes can also be detected in uncultured ovarian tumor cells (Table 2). The expression of the TAG genes in a relatively high frequency of tumors of different tissue origins indicates that TAG-derived antigens would be useful components of vaccines targeting a variety of malignancies.

A frequent goal when immunizing with tumor vaccines is to elicit a tumor-specific CTL response. A common approach to designing such vaccines is to include one or more short antigenic peptides (most often nine amino acids in length) capable of binding to class I MHC molecules. Class I MHC molecules which are prevalent in the population are most often chosen because it maximizes the utility of the vaccine at the population level. For example, by concentrating on the three most prevalent class I MHC molecules in the Caucasian population (i.e. HLA-A1 = 28.1%, A2 = 49.1%, and A3 = 25.0%) (32) coverage for approximately 82% of the population can be obtained. By targeting additional prevalent class I MHC molecules (HLA-B7 = 22.9%, B8=17.9%) it is possible to approach coverage of the entire population. In the present study we used two predictive approaches to identify candidate TAG derived peptides that could be presented by HLA-A1, -A2, -A3, -B7, and –B8 (23, 24).

A challenge presented by using the predictive approach to antigen identification is that different predictive algorithms result in different peptide rankings. By combining the results of two or more predictive algorithms, it is possible to minimize this limitation by focusing on the top ranked peptides from different algorithms, an approach that has been shown to work with both prostate specific membrane antigen (26) and carcinoembryonic antigen (27). We have applied

that approach to the cancer/testis antigen TAG and have attempted to identify new peptide antigens presented in association with HLA-A1, -A2, -A3, -B7, and –B8 (Table 3). Using this approach we tested a total of fifteen peptides that were predicted to bind to one or more of these class I MHC molecules. Of these peptides, four were shown to elicit a response. Of these peptides, LSRLSNRLL was initially shown to be recognized, but as the CTL line eventually lost the recognition of the peptide, it was not studied further.

Two antigenic peptides restricted by HLA-A2 were identified. The SLGWLFLLL peptide was recognized by CTL derived from seven different individuals. This peptide could sensitize targets for lysis with half-maximal killing occurring at between 0.1 and 10 nM which demonstrates that the CTL have a high affinity for the peptide/HLA-A2 complex and makes it likely that the complex could be recognized on the surface of a tumor cell, with such recognition occurring of DM6. Not all HLA-$A2^+$, TAG^+ tumors were recognized by the CTL, however, indicating that either the peptide/MHC complex is present at very low levels on the surface of some tumor cells or that not all tumors positive for TAG expression by PCR express the TAG protein. To begin to address this question we have recently produced recombinant TAG-1 and TAG-2 proteins for the purpose of generating TAG-specific antisera.

The LLLRLECNV peptide stimulated an HLA-A2-restricted response from two of six different donors. The peptide sensitized targets for lysis at 1 to 20 nM, indicating that these CTL had receptors of a slightly lower affinity than those used to recognize the SLGWLFLLL peptide. In contrast to CTL recognizing the SLGWLFLLL peptide, CTL recognizing the LLLRLECNV peptide recognized multiple tumors expressing both HLA-A2 and TAG. Taken together with the results obtained with the SLGWLFLLL peptide-specific CTL, this suggests that the TAG protein is expressed in many tumors positive for TAG expression by PCR and that the LLLRLECNV epitope is selectively expressed over the SLGWLFLLL epitope.

The LPAQEGAPT peptide stimulated an HLA-B7-restricted response in two of three donors and sensitized targets for lysis at concentrations between 20 and 1000 nM which indicates that the peptide is recognized with a relatively low affinity by the elicited CTL. Conversely, the concentration of peptide needed to sensitize targets for half maximal lysis may reflect a poor ability of the peptide to bind to the HLA-B7 molecule. Despite this fact, LPAQEGAPT-specific CTL recognized VMM12 tumor cells which are both C1R-B7$^+$ and TAG$^+$.

It is common to use peptide binding experiments as an intermediate step between the predictive step and the CTL elicitation step, with only peptides demonstrating high affinity binding being selected for further study. The experiments of Lu and Celis (26, 27) have demonstrated, however, that it is possible to dispense with peptide binding experiments when using a predictive approach to peptide epitope identification. Because binding experiments can be both time consuming and expensive to perform, their elimination can greatly streamline antigen identification. The results obtained here confirm that peptide antigens can be successfully identified in the absence of performing preliminary binding experiments. Importantly, our results also demonstrate that the antigen identification process can be further consolidated and made more efficient by simultaneously testing multiple peptides. Although there is a theoretical concern that competition among multiple peptides for binding to a limited number of class I MHC molecules might preclude the identification of an antigenic peptide in a peptide mix, we have successfully used the approach here to identify three different peptides when three or four peptides are included in the mix. Because the *in vitro* stimulations are resource intensive experiments to perform, the ability to simultaneously screen up to four peptides will greatly enhance the utility of the predictive approach to peptide epitope antigen identification.

In combination with a previously identified HLA-A3-restricted TAG epitope (21), a total of four TAG-derived epitopes have now been identified from TAG including two HLA-A2-restricted

epitopes, one HLA-A3-restricted epitope, and one HLA-B7-restricted epitope. Because the TAG family of genes is also expressed in a high percentage of melanomas and in a variety of tumors of epithelial origin, these epitopes are ideal candidates for inclusion in a vaccine for the therapeutic treatment of a variety of malignancies.

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Table 1 Expression of TAG genes in established tumor lines[a]

Tumor Tissue Origin and Type	TAG-1	TAG-2a	TAG-2b	TAG-2c
Tumors of neural origin				
Brain	75% (19-99%)	50% (7-93%)	0% (0-60%)	50% (7-93%)
SW-1088	+	+	-	-
T98G	+	+	-	+
U-87MG	+	-	-	+
U-373MG	-	-	-	-
Tumors of epithelial origin				
Breast	71% (29-96%)	14% (4-58%)	14% (4-58%)	14% (4-58%)
BRC-173	-	-	-	-
BRC-751	+	-	-	-
MCF7	-	-	-	-
MDA-MB-453	+	-	-	-
MDA-MB-468	+++	+++	++	++
SK-BR-3	+	-	-	-
VAB5-A	+	-	-	-
Colon	100% (40-100%)	50% (7-93%)	25% (6-81%)	50% (7-93%)
HT-29	+	-	-	-
LS174T	+	+	-	+
SW480	+	-	+	-
VCR-8	+++	+	-	+
Lung	67% (30-93%)	78% (40-97%)	44 (14-79%)	44 (14-79%)
Calu-1	-	+	-	-
SK-LU-1	+	-	-	-
SK-MES-1	++	++	+	+
TTB-250	-	+	-	-
VBT-2	+	+	-	-
VLU-6	-	-	-	-
VLU-13	+++	+++	+++	+++
VLU-18	+++	+++	++	++
VLU-19	++	+	+	+
Ovarian	82% (48-98%)	55% (23-83%)	18% (23-52%)	27% (6-61%)
CAOV-3	+	+	-	-
CAOV-4	+	-	-	-
COV413	-	+	-	-
ES-2	+	-	-	-
OV-90	+	+	-	-
OVCAR3	+++	+++	++	+++
SK-OV-3	+	-	-	-
SW626	+	+	-	+
TOV-21G	+	-	-	-
TOV-112D	-	-	-	-
TTB-6	+++	+++	++	+++
Pharyngeal	100% (2-100%)	0% (0-98%)	0% (0-98%)	0% (0-98%)
FaDu	+	-	-	-
Tongue	100% (2-100%)	0% (0-98%)	0% (0-98%)	0% (0-98%)
SCC4	+	-	-	-
Cervical	0% (0-98%)	0% (0-98%)	0% (0-98%)	0% (0-98%)
SiHa	-	-	-	-

[a]PCR was performed as described in the Materials and Methods. The PCR products were visualized on ethidium bromide-stained agarose gels and the staining intensity ranked as: +++, product was easily visualized after 30 cycles of amplification; ++, product was weakly visible at 30 cycles and easily visible after 40 cycles of amplification; +, product was only visible following 40 cycles of amplification; -, product was not visualized following 40 cycles of amplification. The percentage positive with 95% confidence intervals is given for each tumor type. Each sample exhibited a strong signal when GAPDH was PCR amplified.

Table 2 Expression of TAG genes in uncultured melanoma and ovarian carcinoma cells

Sample Numbers	Gene Expression Pattern[a]			
	TAG-1	TAG-2a	TAG-2b	TAG-2c
Melanoma				
204, 415, 7719	++	++	+	++
956	++	++	+	+
2241	++	++	-	++
8062	++	+	-	-
3540	+	+	+	+
1302	+	+	-	-
278, 550, 1435, 2348, 8353	+	-	-	-
123, 243, 482, 509, 2201, 4479, 8326, 8542, 8899	-	-	-	-
Total Positive	13/22	8/22	5/22	6/22
% Positive	59%	36%	23%	27%
95% Confidence Interval	36-79%	17-59%	8-45%	11-50%
Ovarian Carcinoma				
6, 519	++	++	+	++
144, 632	+	+	-	-
117, 121, 189, 1006	+	-	-	-
1130	-	+	-	-
29, 94, 125, 136, 185, 212, 227, 258, 546, 567, 572, 834, 1288, 3883	-	-	-	-
Total Positive	8/23	5/23	2/23	2/23
% Positive	35%	22%	9%	9%
95% Confidence Interval	16-57%	8-44%	1-28%	1-28%

[a]PCR was performed as described in the Materials and Methods. Following 30 rounds of amplification the PCR products were visualized on ethidium bromide-stained agarose gels and the staining intensity ranked as: (++) product was easily visualized; (+) product could be visualized, but the band was very light; (-) product was not visible. Each sample exhibited a strong signal when GAPDH was PCR amplified.

Table 3 Test peptides from TAG used for *in vitro* CTL priming

Class I MHC Binding Protein[a]	Peptide Sequence[b]	Residue Numbers[c]	Presence in TAG-1 or TAG-2	Parker Score[d]	SYFPEITHI Score[e]
HLA-A1	ES<u>E</u>RGLPAS	32-40	1, 2	0.27	16
	NL<u>E</u>PLVSRD	64-72	1, 2	0.90	16
	SR<u>D</u>PPASAS	70-78	1, 2	0.25	17
HLA-A2	T<u>L</u>SRLSNR<u>L</u>	41-49	1, 2	21.4	22
	<u>L</u>LLRLECN<u>V</u>	49-57	1, 2	487.5	25
	S<u>L</u>GWLFLL<u>L</u>	78-86	1	40.6	24
	F<u>L</u>LLLNSTT	83-91	1	126.8	20
HLA-A3	G<u>L</u>PASTLS<u>R</u>	36-44	1, 2	24.0	21
	<u>L</u>LLLNSTT<u>K</u>	84-92	1	30.0	28
HLA-B7	L<u>P</u>AQEGAPT	1-9	1, 2	2.0	20
	VQ<u>R</u>RAEGL<u>L</u>	10-18	1, 2	40.0	12
	L<u>P</u>ASTLSR<u>L</u>	37-45	1, 2	80.0	21
	LS<u>R</u>LSNRL<u>L</u>	42-50	1, 2	40.0	12
	D<u>P</u>PASASLF	72-80	2	0.4	11
HLA-B8	TVQR<u>R</u>AEGL	9-17	1, 2	4.0	18
	VQ<u>R</u>RAEGLL	10-18	1, 2	1.2	17
	LS<u>R</u>LSNRLL	42-50	1, 2	4.0	18

[a] Antigenic peptides from TAG-1 and TAG-2 were predicted based on the predicted ability of the peptides to bind to the indicated class I MHC molecule.

[b] Underscored residues correspond to canonical amino acid residues typically found at that position of a peptide binding to the respective class I MHC molecule.

[c] Position of the peptide within the linear sequence of TAG-1 and TAG-2.

[d] Score obtained from predictive algorithm of Parker (24).

[e] Score obtained from the predictive algorithm of Rammensee (23).

Table 4 Summary of CTL reactivity against stimulating peptides

Class I MHC Binding Protein	Stimulating Peptide(s)[a]	No. of positive cultures obtained/No. of cultures initiated[b]	Specificity of obtained CTL[c]
HLA-A1	SRDPPASAS	0/6	NT
HLA-A1	ESERGLPAS NLEPLVSRD	0/6	NT
HLA-A2	SLGWLFLLL	7/9	SLGWLFLLL
HLA-A2	SLGWLFLLL TLSRLSNRL LLLRLECNV FLLLLNSTT	1/1	SLGWLFLLL LLLRLECNV
HLA-A2	TLSRLSNRL LLLRLECNV FLLLLNSTT	1/5	LLLRLECNV
HLA-A3	GLPASTLSR	0/6	NT
HLA-A3	LLLLNSTTK	0/6	NT
HLA-B7	LPASTLSRL	0/6	NT
HLA-B7	LPAQEGAPT VQRRAEGLL LSRLSNRLL DPPASASLF	2/3	LPAQEGAPT
HLA-B8	LSRLSNRLL	1/9	LSRLSNRLL
HLA-B8	TVQRRAEGL VQRRAEGLL	0/6	NT

[a]Peptide priming of CTL was performed as indicated in the Materials and Methods, and was done with either a single peptide or a pool of two to four peptides as indicated.

[b]A culture was considered positive if the responder cells in at least one well from a given donor recognized target cells pulsed with the corresponding peptide(s) in both initial screening assays.

[c]Initial screening for positive cultures used target cells pulsed with a pool of peptides when a peptide pool was used for the stimulations. Once a CTL line was established, it was tested against target cells pulsed with the individual peptides.

Figure Legends

Fig. 1 Peptide reactivity of anti-CD3 expanded, SLGWLFLLL (SLG) peptide-stimulated microcultures. Expanded microcultures were tested in a ^{51}Cr-release assay against the indicated targets. Six independent cultures are shown, each derived from a separate donor. C1R-A2 and T2 are HLA-A2^{+} targets.

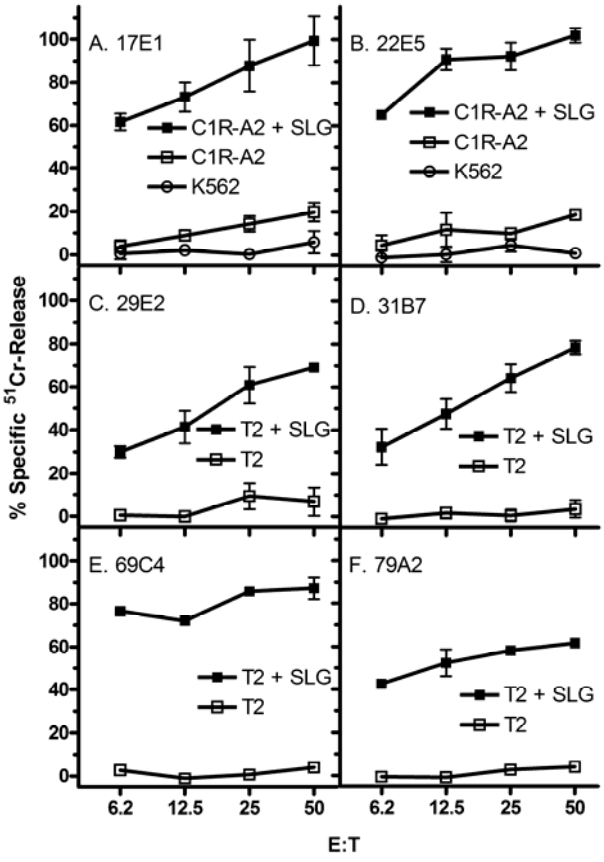

Fig. 2 Peptide specificity of peptide mix-stimulated microcultures. Microcultures 82C8 (A) and 65F2 (B) were tested in a ^{51}Cr-release assay against targets expressing either a mix of peptides predicted to associate with HLA-A2 (A) or HLA-B7 (B). Following expansion with anti-CD3, 82C8 (C) and 65F2 (D) were tested against the individual peptides which comprised the original mix. T2 is an HLA-A2^{+} target; C1R-B7 is an HLA-B7^{+} target. Peptides used were FLLLNSTT (FLL), LLLRLECNV (LLLR), TLSRLSNRL (TLS), SLGWLFLLL (SLG), DPPASASLF (DPP), LPASTLSRL (LPA), LSRLSNRLL (LSR), and VQRRAEGLL (VQR).

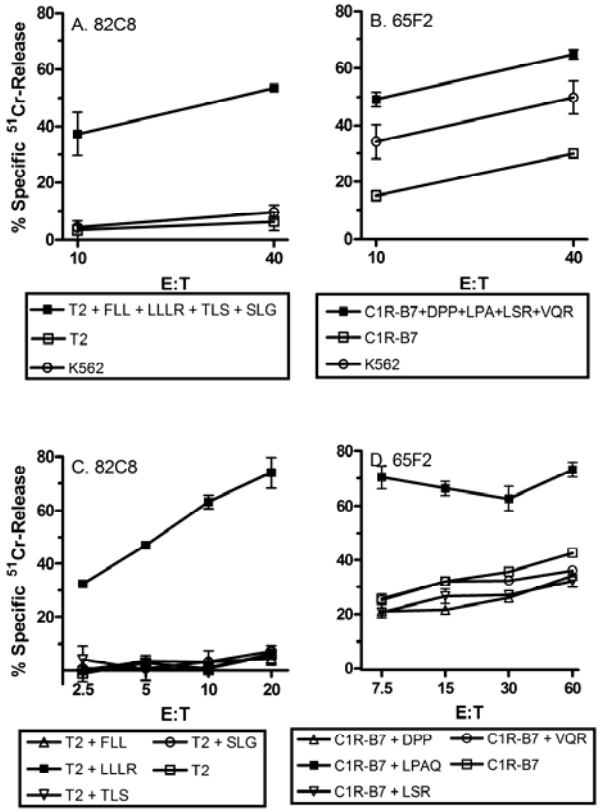

Fig. 3 MHC restriction of the SLGWLFLLL (SLG), LLLRLECNV (LLLR), and LPAQEGAPT (LPAQ) peptides. CTL lines 22E5 (A), 82C8 (B), and 65F2 (C) were tested in a ^{51}Cr-release assay for their ability to kill the indicated targets. Solid symbols indicate that the targets cells were pulsed with 2.5 µg/ml of the indicated peptide as described in the Materials and Methods; open symbols indicate that the targets were not pulsed with peptide. Solid lines indicate that the targets are HLA-A2$^+$ (A, B) or HLA-B7$^+$ (C); dashed lines indicate that the targets are HLA-A2$^-$ (A, B) or HLA-B7$^-$ (C).

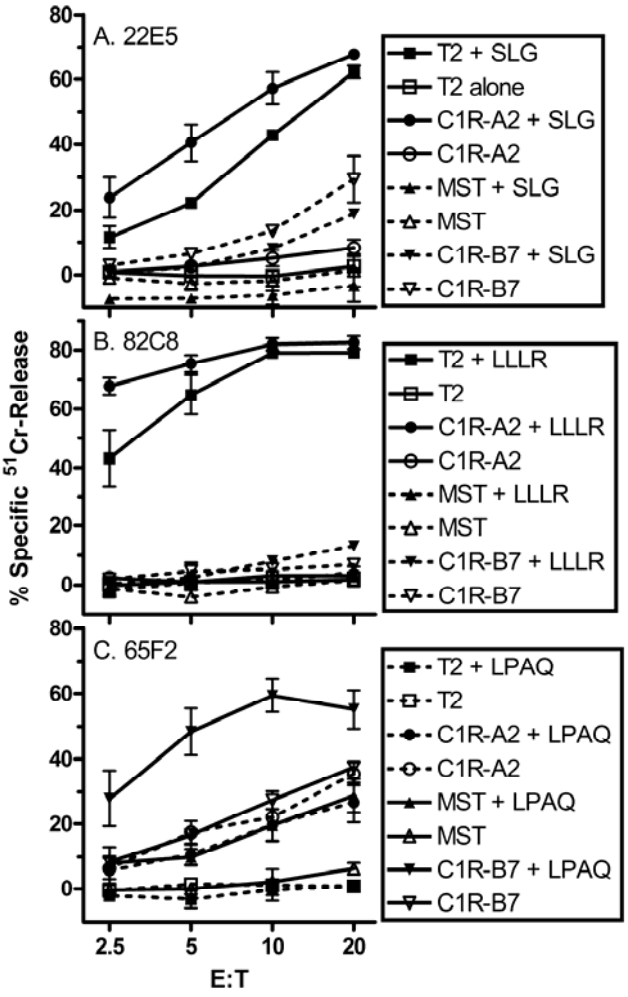

Fig. 4 Peptide dose-response of peptide-specific CTL. The indicated target cells were pre-incubated with peptide at the indicated concentrations for 1 hr at 37°C and then used as targets in a standard ^{51}Cr-release assay with the indicated CTL lines. CTL were used at an E:T of 5:1 except line 65F2 which was used at an E:T of 10:1. The peptides used were SLGWLFLLL (SLG), LLLRLECNV (LLLR), and LPAQEGAPT (LPAQ).

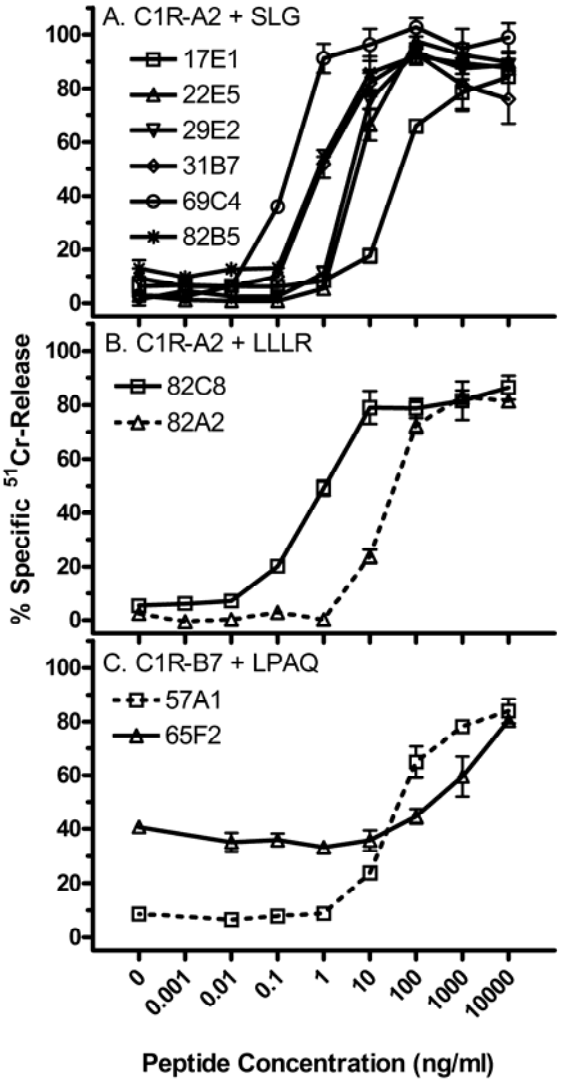

Fig. 5 Recognition of tumor lines by peptide-specific CTL. CTL lines 22E5 (A), 69C4 (B), 65F2 (C), and 82C8 (D) were tested in a standard ^{51}Cr-release assay against the indicated targets. Open symbols indicate that the target was incubated with the cognate TAG peptide or expresses the TAG gene; closed symbols indicate that the target was neither incubated with the cognate TAG peptide nor expressed the TAG gene. Solid lines indicate that the target cells are matched with the CTL for expression of HLA-A2 (A, B, D) or HLA-B7 (C); dashed lines indicate that the target cells do not share HLA-A2 or HLA-B7 in common with the CTL (A-D). The peptides used were SLGWLFLLL (SLG), LLLRLECNV (LLLR), and LPAQEGAPT (LPAQ).

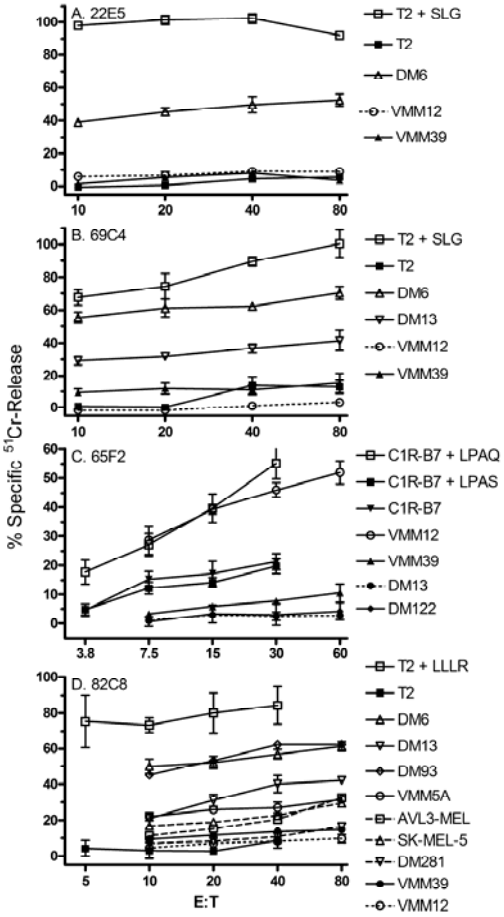

Fig. 6 Cold target inhibition analysis of peptide-specific CTL. CTL were pre-incubated for one hour at 37°C with the indicated ratio of cold targets, after which the hot targets were added and incubation continued for an additional four hours. CTL line 22E5 was used at an E:T of 10:1 (A), CTL line 82C8 was used at an E:T of 40:1 (B), and CTL line 65F2 was used at an E:T of 30:1 (C). Peptides used were GILGFVFTL (GIL), SLGWLFLLL (SLG), LLLRLECNV (LLLR), LPASTLSRL (LPAS), and LPAQEGAPT (LPAQ).